Biotechnol Lett (2006) 28:1493–1501
DOI 10.1007/s10529-006-9119-2

ORIGINAL PAPER

Wound and insect herbivory responsive genes in poplar

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Received: 22 May 2006 / Accepted: 26 May 2006 / Published online: 1 August 2006

Abstract Insect herbivory leads to induced resistance to subsequent infestations in plants. This is due in part to feeding-induced expression of genes that can lead to reduced palatability and/or digestibility of the plant material. We identified 57 distinct differentially expressed genes from poplars that were either infested by gypsy moth (*Lymantria dispar*) or mechanically wounded. Eleven highly insect-inducible genes were also found to be wound-inducible. Time course analysis revealed diverse timing of peak transcript accumulation. Sequence analysis of promoters suggested that the wound responsive elements, W and DRE, and the jasmonic acid responsive H motif, are over-represented in wound-induced poplar promoters and should be investigated further.

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Keywords Defense · DRE-box · H-box · Herbivory · Infestation · Poplar · *Populus* · W-box · Wounding

Introduction

Plants have evolved numerous responses to infestation by insects. These can include defensive as well as physiological changes that are thought to be a consequence of shifts in carbon and nitrogen partitioning within plant cells. Defensive changes can be directly deleterious to the insect such as the production of secondary metabolites or proteinase inhibitors. Alternatively, indirect defenses involve the creation of volatile compounds that act to direct herbivore predators to the site of infestation (for review see Kessler and Baldwin 2002). Defensive responses can inhibit pest attack and/or make the plant less preferable to insect herbivores.

Differential display and microarray technology have shown that feeding by chewing insects results in many changes in gene expression (Reymond et al. 2000, 2004; Arimura et al. 2000; Hermsmeier et al. 2001, Hui et al. 2003). In *Nicotiana attenuata*, infestation by *Manduca sexta* down-regulated photosynthetic genes; while genes involved in stress, wounding and pathogens, and those associated with shifts of carbon and

nitrogen, were up-regulated (Hermsmeier et al. 2001).

Resistance to insects in *Populus* includes induced responses causing reduced performance by future insect herbivores (Mattson and Palmer 1988; Clausen et al. 1989; Robison and Raffa 1997; Havill and Raffa 1999). Many individual genes have been identified as wound or infestation induced in poplar, for example, 20 systemically wound induced genes were found by Chistopher et al. (2004) using macroarray analysis of systemically wound-induced cDNAs. Recently a 15,000 cDNA microarray of hybrid poplar representing 25% of the genome was examined and 1728 genes responsive to forest tent caterpillar damage over 24 h were identified (Ralph et al. 2006). This work provides a valuable resource to begin evaluating plant–insect interaction in a forest tree.

Wounding is often used to mimic insect infestation since it is easier to standardize the treatment. Analysis of wound responsive promoters has allowed numerous motifs to be identified. Rushton et al. (2002) verified that these motifs confer wound-induced expression by testing synthetic promoters containing the elements in *Arabidopsis*. They found that 4 copies of the W, GCC, S, JERE, DRE and GST motifs in a background of a minimal 35S promoter each conferred wound-induced expression of β-glucuronidase. In addition, the G box, GCC box, H box and TGA motif can be found in jasmonic acid (JA) responsive genes, which are generally associated with the wound response (Mason et al. 1993; Nishuichi et al. 2004; Takeda et al. 1999; Rouster et al. 1997). The W box has been found in a number of wound-responsive genes (Hara et al. 2000).

We have used differential RNA display to catalog genes that respond to wounding and infestation in poplar. Wound- and infestation-induced expression was confirmed by either northern or reverse northern analyses. Promoter sequence analysis suggested enrichment of specific types of cis-elements that warrant further study. The gene catalog reported here assigns putative roles in defense to >50 genes in an ecologically important plant system.

Materials and methods

Plant and gypsy moth materials

Poplars (*Populus trichocarpa* Torr. and Gray × *P. deltoides* Bartr. ex Marsh. hybrid H11-11) were grown in 3.7 l pots at 23°C under 12 h light /12 h dark cycles and grown to a height of 60 cm. Gypsy moth (*Lymantria dispar*) were obtained from USDA-Aphis (Otis Intl. Guard Base, MA) and maintained at 25°C under 12 h light/12 h dark cycles on artificial diet.

Infestation experiments

Third and fourth instar gypsy moth larvae were held for 2 d on detached poplar branches placed in water-filled bottles, then starved for 21 h so that insects would be receptive to feeding. Sylleptic branches were covered with a fine mesh sleeve, 25 gypsy moth larvae were added and branches were infested for 1 h, after which, larvae were removed and all the leaves per sylleptic branch were harvested 3 h later. Sylleptic branches on the same plant were used as controls. Mechanical wounding was performed by applying ~15 plier ''bites'' per first mature leaf outside the veins, at 0, 2 and 4 h and the leaves harvested at 6 h. In time course experiments, the first fully expanded leaf was wounded using ~15 plier bites then harvested immediately, or 0.25, 0.5, 1, 2 and 6 h after injury. Infestation and mechanical wounding was not meant to mimic each other quantitatively.

Molecular analysis

Leaf material was harvested and rapidly frozen in liquid N_2. RNA was isolated as described by Chang et al. (1993). Differential display of mRNA (Liang and Pardee 1992)) was carried out according to the manufacturer's instructions (GenHunter, Nashville TN). DNase-treated RNA was used for first strand cDNA synthesis by MMLV-RT (GibcoBRL, Gaithersburg, MD). Bands were selected if differentially expressed by wounding or infestation. The cDNA was amplified via PCR with 7.4 KBq/µl α-[^{33}P]dATP

included in the reaction mixture. PCR products were separated by 6% denaturing polyacrylamide gels (HR1000, Genomyx, Foster City, CA). The bands of interest were excised from the gel, reamplified by PCR, and ligated to pGEM-T (Promega, Madison, WI) for transformation into *E. coli* DH5α (GibcoBRL, Gaithersburg, MD). DNA was sequenced on a model 310 Genetic Analyzer using dye terminator reactions from PE Applied Biosystems (Foster City, CA) and analyzed for similarity to known genes using BLAST (Altschul et al. 1997).

Membrane arrays were performed, probed and analyzed as described in Cooke et al. (2003). The membrane arrays were used to characterize the most strongly infestation- induced genes. Array probes were labeled first strand cDNA from leaves subjected to either gypsy moth herbivory for 24 h (3 membranes × 2 spots/cDNA/membrane), mechanical injury after ~15 plier ''bites'' per leaf applied at 0, 2 and 4 h and harvested at 6 h (4 membranes × 2 spots/cDNA/membrane), or corresponding undamaged control leaf samples (3 and 4 membranes with 2 spots/cDNA/membrane for insect and wounding, respectively). cDNA probes were biologically replicated as well. Volumes (transcript abundance) for each cDNA were obtained from a Storm phosphorimager (Molecular Dynamics), using the local background subtraction option in ImageQuant software, and the two spots were averaged to generate a single cDNA mean for each membrane. Poor quality spots were identified visually and eliminated from the analysis. All volumes on a particular membrane were divided by the volume of cab (chlorophyll a/b binding protein gene) on that same membrane. *t*-tests and ANOVA were carried out using Excel and SAS (SAS Institute, Cary, NC).

Database, wound associated motifs and phylogenetic analysis

Comparisons to the National Computer Biological Initiative (NCBI) database were made using BLASTX (Altschul et al. 1997). Poplar wound-induced genes were identified by BLASTN analysis of the poplar genome at http://genome.jgi-psf.org/Poptr1/Poptr1.home.html. Consequently the gene regions are from the Nisqually 1 genotype. A total of 1.5 kb upstream of the apparent translation start codon was extracted for 15 confirmed highly wound-induced poplar genes. Motif frequencies in random promoter sequences were calculated based on observed A/T (0.35) and G/C (0.15) content in these promoter sequences. Protein sequences were aligned using Megalign of DNAStar. Phylogenetic analysis was performed using the parsimony option of PAUP (Swofford 1998).

Results and discussion

Genes induced by herbivory or wounding

Using differential display, a total of 175 cDNA clones were isolated that were induced by herbivory or wounding. These 175 cDNA picks (27 from infested vs. control comparisons; 148 from wound vs. control comparisons) coalesced into 126 contigs (22 from infested vs. control comparisons; 104 from wound vs. control comparisons) based on sequence identity. This disparity was probably due to the larger number of primer pairs tested with the wound-induced material. The reverse transcription reaction in differential display involves a primer that binds the poly A tract of mRNAs, so that there is a tendency to clone 3′ ends of transcripts. Upon comparison of the identified clones with the NCBI database, 45% were found to encode proteins with presumed function already present in the database (Table 1). Clones with similarity to known proteins were categorized according to putative function (Table 1). Defense-related genes were present 25% of the time, as were genes involved in metabolism. Stress-responsive genes made up 15% of the clones. Sequences that could not be definitively categorized but belonged in either metabolism or defense (2%) and metabolism or stress (8%) were also present. Regulatory (10%), cell wall (6%), cytoskeletal (4%) and developmental (4%) genes made up the remaining groups.

Membrane arrays were performed and the eleven cDNAs most highly expressed in insect-infested leaves were identified (4–33 fold) compared to untreated controls (Fig. 1). All of these

Table 1 Identification of cDNA clones isolated from infestation and wounding differential display. Categories; SR = stress, CS = cytoskeletal, Dev = developmental, Def = Defense, Met = metabolism, CW = cell wall, Reg = regulatory

Genbank #	Similarity	Category	Blast-P	Lab ID
EB710343	RZ403 (AA231867)	plantEST	5.40E-21	PO 34
EB710344	Thioredoxin M (CAA06735)	Def	3.00E-14	PO 37
EB710345	Formaldehyde dehydrogenase (S71244)	Met	3.00E-40	PO 38
EB710346	Hydroxyproline-rich protein (AAD01800.1)	Dev	8.00E-14	PO 164A
EB710347	40S Ribosomal protein (P49198)	Met	1.00E-10	PO 167
EB710348	Trypsin inhibitor (AAA68962.1)	Def	2.00E-47	PO 175
EB710349	26S Proteasome subunit (BAA97246.1)	Reg	2.00E-47	PO 176
EB710350	Alpha tubulin (AAD50627.1)	CS	4.00E-07	PO 178
EB710351	Microtubule-assoc. protein (AAD24645.1)	CS	1.00E-19	PO 179
EB710352	Oxysterol-binding protein (BAA97478.1)	SR	5.00E-46	PO 185
EB710353	Gn-c1042-273 5' (BE348131.1)	plant EST	6.00E-10	PO 190
EB710354	Trehalose-6-phosphate synthase (AAF16560.1)	Def	2.00E-12	PO 196
EB710355	pop3 (T09703)	Def	7.00E-15	PO 197
EB710356	Lipoxygenase LoxC (AJ002236)	Def	2.20E-09	PODD7
EB710357	Putative protein (AL021711)		1.20E-13	POT 37
EB710358	40S Ribosomal protein S5 (Y08860)	Met	1.10E-27	POT 38
EB710359	Glycerophosphodiesterase (1399038)	Met/SR	1.00E-170	POT 41
EB710360	Isoflavone reductase like protein (Y12689)	SR	1.60E-51	POT 44
EB710361	Cytokinin-regulated kinase 1 (AAG25966.1)	Reg	5.00E-07	POT 52
EB710362	Chorismate synthase 1 (Q42884)	Met	2.00E-51	POT 56
EB710399	Putative RNA polymerase (3894190)	Met	2.00E-08	POT 100
EB710363	SUMO conjugating enzyme (2267139)	Reg	1.00E-29	POT 103
EB710364	Unknown protein (3241945)		4.00E-21	POT 108
EB710365	IAA amidohydrolase (AJ005340)	Dev	3.00E-07	POT 113
EB710366	Acidic four domain chitinase (403416)	Def	2.00E-05	POT 124
EB710367	Lectin (P83304)	Def	3.00E-8	POT 134
EB710368	Lectin-nucleotide phosphohydrolase (AAD31285.1)	Def	1.00E-20	POT 136
EB710369	Glutathione *S*-transferase (2920666)	Met/SR	9.00E-12	POT 137
EB710370	3-ketoacyl-CoA thiolase (BAA11117)	Met	4.00E-37	POT 138
EB710371	Unknown protein (AAD22119.1)		1.00E-12	POT 146
EB710372	Hydroperoxide lyase (3822403)	Def	3.00E-25	POT 151
EB710373	Sn04c03.y1 (BE057580.1)	plantEST	5.00E-07	POT 156
EB710374	Phosphoglycerate kinase (AB018412.1)	Met	7.00E-96	POT 157
EB710375	Tryptophan synthase beta-subunit (P25269)	Met	2.00E-50	POT 158
EB710376	Blue copper binding protein (Q07488)	CW	1.00E-24	POT 159
EB710377	Glutathione *S*-transferase (Y13898)	Met/SR	8.00E-14	POT 160
EB710378	Cinnamyl dehydrogenase (NP195643)	CW	1.00E-113	POT 165
EB710379	Unknown protein (AAD24599.1)		4.00E-21	POT 166
EB710380	Inducible GST (920666)	Met/SR	7.00E-44	POT 169
EB710381	Glyoxalase (AB017042)	SR	7.00E-41	POT 170
EB710382	Caffeoyl CoA 3 O-methyltransferase (AJ224895)	CW	3.00E-75	POT 171
EB710383	est (AI163598)		2.00E-58	POT 174
EB710384	Ribosomal protein S12 (O13019)	Met	3.00E-26	POT 179
EB710385	Cytochrome f precursor (P06449)	Met	8.00E-54	POT 181
EB710386	Hypothetical protein (S57813)		2.00E-09	POT 186
EB710387	Legumin 11S globulin (949867)	Met	2.00E-13	POT 187
EB710388	Protease regulatory subunit 6	Reg	3.00E-71	POT 194
EB710389	Allene oxide synthase (P48417)	Def	8.00E-48	POT 195
EB710390	Trypsin inhibitor win3 (X15516)	Def	2.00E-04	POT 197
EB710391	F25C20.9 (AAD30247.1)	plantEST	2.00E-12	POT 199
EB710392	Heme oxygenase (AAD22107.1)	SR	3.00E-18	POT 200
EB710393	Glycine cleavage protein H (P93255)	SR	3.00E-34	POT 201
EB710394	Thioesterase like protein (CAB10528.1)	Met	2.00E-10	POT 212
EB710395	Glutathione *S*-transferase (4056432)	SR	2.00E-26	POT 215
EB710396	Cysteine proteinase inhibitor (BAA19608)	Def	1.00E-16	POT 221

Table 1 continued

Genbank #	Similarity	Category	Blast-P	Lab ID
EB710397	hsp20 (AJ225046)	SR	1.00E-15	POT 226
EB710398	3-PI-dependent protein kinase (AY062684.1)	Reg	4.00E-10	POT 228

genes were also induced by mechanical injury although the magnitude of the response was not highly correlated. Reymond et al. (2004) identified 39 genes from *Arabidopsis* that were induced by infestation with both a generalist and specialist insect pest but not after mechanical wounding suggesting that responses to insect infestation and wounding are mediated by partially overlapping signal transduction pathways.

Jasmonic acid (JA) biosynthesis has been implicated as a control mechanism in plant wound responses. JA is an ocatadecanoid lipid-derived signal that is similar in structure to prostaglandins and leukotrienes found in animals. Sequence analysis suggests that we identified two genes that encode enzymes in the octadecanoid pathway, allene oxide synthase (AOS; Pot 195) and hydroperoxide lyase (HPL; Pot 151). These enzymes represent a unique class of cytochrome P450s specialized for the metabolism of fatty acid hydroperoxides (Song et al. 1993). AOS and HPL both use 13*S*-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid (13-HPOT) as a substrate, and this homology is reflected by sequence similarity of the genes. A gene tree further supported our inference that Pot 195 encodes AOS whereas Pot 151 encodes HPL (Fig. 2).

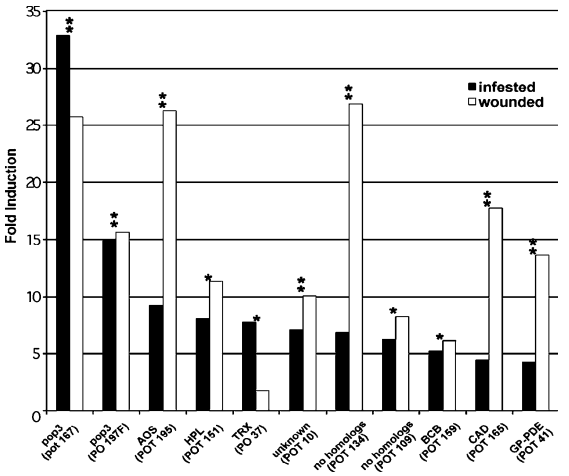

Fig. 1 Genes induced by insect herbivory are also induced by wounding. Fold induction of genes strongly induced by insect herbivory and mechanical wounding. One tailed *t*-test was performed since genes were expected to be induced. Wounding or infestation were compared to their respective controls (*, $P < 0.05$; **, $P < 0.01$). Most closely associated gene name is shown with the lab ID. Pop3 is also known as the boiling stable protein; AOS, allene oxide synthase; HPL, hydroperoxide synthase; TRX, thiredoxin; BCB, blue copper binding protein; CAD, cinnamyl aldehyde dehyrogenase; GP-PDE, glycerophosphodiesterase

We selected fourteen cDNAs representing a variety of cellular processes including JA biosynthesis and used them as probes in a time course experiment to determine relative timing of wound-inducible gene expression. All of the genes were wound-inducible and showed a continuum of transcript accumulation with respect to the timing of transcript detection, peak accumulation and decay (Fig. 3). Most transcripts were detected 0.5 or 1 h after wounding. The most rapid wound response was detected for a gene that is conserved in plants and animals but has unknown function (Pot 10). Transcripts encoding SUMO conjugating enzyme, IAA amidohydrolase, glyoxalase, a ribosomal protein and a cysteine proteinase inhibitor were at or near maximum levels at 2 h after wounding. Glyoxalase confers stress tolerance (Veena et al. 1999) while cysteine proteinase inhibitors are thought to inhibit insect feeding. Transcripts for glycerophospho-diesterase, allene oxide synthase, lipoxygenase and hydroperoxide lyase appeared to be coordinately regulated and the latter three enzymes play known roles in the biosynthesis of JA downstream of linolenic acid. Glycerophosphatediesterase produces glycerol 3-phosphate, potentially to recycle G3P during lipid catabolism. Transcripts for chitinase, acetyl coA acyltransferase, isoflavone reductase and glutathione *S*-transferase continued accumulation through

6 h. Recombinant poplar chitnase inhibits Colorado potato beetle development (Lawrence and Novak 2006), acetyl coA acyltransferase is involved in lipid metabolism, isoflavone reductase is induced by elicitors and glutathione *S*-reductase is induced by pathogen attack or during detoxification.

Based on inference from the transcript abundance data, induction of genes involved in JA biosynthesis appears to be a prominent feature of the wound response in poplar, as it is for other plants. It is tempting to speculate that JA biosynthesis may be more important for regulation of genes induced later than it is for extremely early genes (e.g. Pot 10).

AOS is the first committed step in the synthesis of jasmonic acid, which is involved in a number of physiological processes including wound or herbivore induced signaling (Turner et al. 2002). Jasmonic acid is produced locally in response to these perturbations and induces defense related genes (Howe et al. 1996; McConn et al. 1997). HPL generates C6 aldehyde compounds that form the volatile ''green note'' flavor given off by damaged leaf tissue and a C12 compound leading to the generation of the wound signal traumatin (Bate et al. 1998). The C6 compounds may also be involved in the induction of a subset of defense related genes (Bate and Rothstein 2001). Antisense HPL (as-HPL) transgenic potato plants have been shown to increase fecundity, population sizes and decrease time to development of

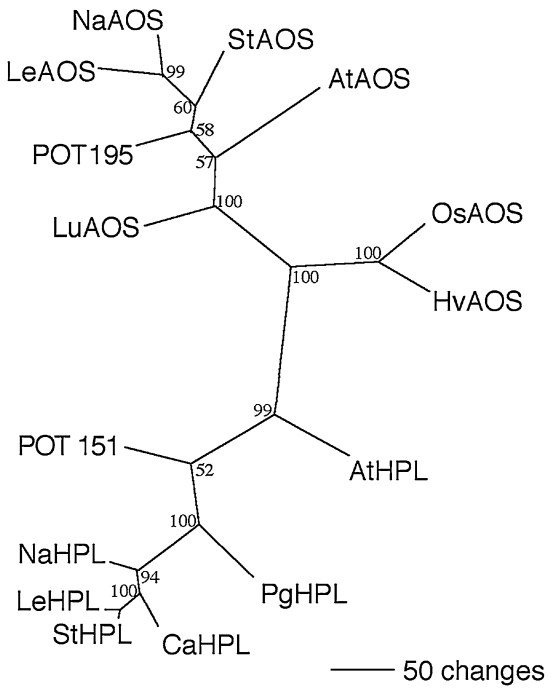

Fig. 2 POT 151 is most closely related to HPL homologs, while POT 195 associates with homologs of AOS. Phylogenetic analysis was performed using the parsimony option of PAUP. Boot strap analysis is shown at the nodes. CaPHL, *Capsicum annum*, red pepper, AAA97465; StHPL, *Solanum tuberosum*, potato, CAC44040; LeHPL, *Lycopersicon esculentum*, tomato, AAF67142; NaHPL, *Nicotiana attenuata*, CAC91565; PgHPL, *Psidium guaiaya*, guava, AAK15070, AtHPL, *Arabidopsis thaliana*, NP_193279; HvAOS, *Hordeum vulgare*, barley, CAB86384; OsAOS, *Oryza sativa*, Japanese rice, AAL38184; LuAOS, *Linum usitatissimum*, flax, AAA03353; AtAOS, *Arabidopsis thaliana*, CAA63266; StAOS, *Solanum tuberosum*, potato, AJ457080.1; NaAOS, *Nicotiana attenuata*, CAC82911; LeAOS, *Lycopersicum esculentum*, tomato, AAF67141

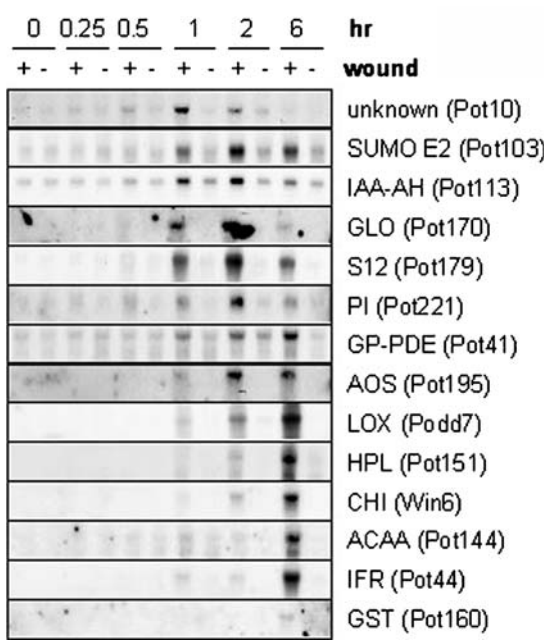

Fig. 3 Early versus late induction of a subset of wound-induced genes. A Northern blot of a wounded (W) or unwounded (U) transition leaf was harvested from zero to 6 h after treatment. SUMO E2, SUMO conjugating enzyme; IAA-AH, IAA amidohydrolase; GLO, glyoxalase; S12, ribosomal protein S12; PI, cysteine proteinase inhibitor; GP-PDE, glycerophosphodiesterase; AOS, allene oxide synthase; LOX, lipoxygenase; HPL, hydroperoxide lyase; CHI, chitinase; ACAA, acetyl CoA acetyl transferase; IFR, isoflavone reductase like protein; GST, glutathione *S*-transferase

Myzus persicae aphids (Vancanneyt et al. 2001). Although recently, Halitschke et al. (2004) showed that *Manduca sexta* developed more slowly and consumed less leaf area on as-HPL plants. They concluded that the reduction of green leaf volatiles (GLV) found in as-HPL plants acts as a feeding stimulant since addition of GLV to as-HPL plants restored wildtype levels of leaf consumption and *Manduca* development. Therefore, reduction of HPL does not have a predictable effect on plant–insect interactions and may be dependent on the specific evolutionary relationship of the plant–insect partners.

Promoter elements found in mechanically wound-induced genes

Since the poplar genome has been sequenced, it is possible to examine the region 5′ of the coding sequence of wound-induced genes in an attempt to identify potential binding sites for regulators of inducible transcription. The 1.5 kb region 5′ of the ATG start codon was examined for biotic and abiotic stress-related motifs in 15 wound-induced poplar genes. Genes were chosen based on confirmation of wound-induction by northern analysis and by both similarity to other cDNAs in the database and the presence of the initiator codon in the cDNA. Three motifs appear to be over-represented in the wound-induced genes (Table 2). The DRE box, the W box and the H box appear to be enriched in the wound-induced genes. DRE elements were named drought responsive elements because they were associated with genes that are induced by water stress. W box elements are recognized by WRKY transcription factors. H boxes are found in promoters responsive to jasmonate. Three promoters are particularly notable and worthy of further study. The isoflavone reductase promoter (Pot 44) has 10 DRE elements. The AOS promoter (Pot 195) has 2 DREs and 3 W-box motifs. One of the glutathione S-transferase genes (Pot 160) has 4 W-box motifs. To our knowledge this is the first analysis of multiple wound-induced promoters in poplar. Our results suggest motifs within promoters that could be involved in the wound response in poplar. Mutation or deletion of these motifs in individual promoters using reporter gene fusions could be performed to test the roles of these motifs. A more comprehensive scan of the poplar wound-regulated transcriptome using whole-genome microarrays may also be a useful tool to test co-regulation of genes with similar promoter motifs.

Table 2 Observed and expected frequencies of motifs in poplar wound-induced promoters

Motif	Observed in 15 wound promoters	Expected in 15 random sequences
W box (TTGACC)	18	6.5
G box (CACGTG)	4	2.8
GCC box (GCCGCC)	1	0.5
TGA (TGACG)	15	18.6
S (AGCCACC)	1	0.4
DRE (CCGAC)	27	8.0
H box (CCATCC)	9	2.8

The sequence of the poplar genome is complete, which makes it the ideal tree species to examine a genomic response to insect herbivory. Cloning of herbivory responsive promoters in poplar involves simple PCR procedures. The large leaf size makes isolation of locally and systemically induced material straightforward when compared with the model organism *Arabidopsis*. Reliable tissue culture protocols allow questions to be asked using reverse genetics.

The genes we have described as herbivory responsive in poplar are generally associated with defense. Producing jasmonic acid and GLV may lead to direct or indirect defense reactions. The availability of the poplar genome sequence should enhance opportunities to define the functions of inducible genes in conditioning resistance to insect herbivory in poplar trees.

Acknowledgements We thank Kevin Thorpe for providing the gypsy moth larvae. We appreciate the efforts of Dawn Gundersen-Rindal and Jeffrey Slack for their critical reading of the manuscript.

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